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Chemical and Physico-Chemical Characterization of Genetic Variant D of Bovine β-Lactoglobulin

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The recently discovered genetic variant of β-lactoglobulin, β-D, has been compared to those previously known. It has been shown that β-D varies from β-B by the substitution per subunit chain of a glutamine residue in β-D for glutamic acid in β-B. This conclusion has been verified by titration experiments. Conformational studies have shown that the structure of β-D is indistinguishable from that of the other variants. Furthermore, β-D does not form the low temperature octamer, characteristic of the A variant, while it dissociates into subunits both at acid and alkaline pH, just as the other variants.

Until recently, three genetic variants of bovine β-lactoglobulin had been identified. Variant A differs from B by the amino acid replacements A (Asp, Val) ↔ B (Gly, Ala) and C differs from B by the replacement C (His) ↔ B (Gln) per polypeptide chain (1–3).

These three proteins appear to have similar secondary and tertiary structures and the changes which occur in their conformations when the pH is varied are essentially identical in all three (4). Between pH 6.5 and pH 9.5, they all undergo the same transition, which involves a single abnormal carboxyl per polypeptide chain (5).

Besides differences in their net charge, however, and their different antigenic behavior (6), the three variants display some differences in their physico-chemical properties. The transition between pH 4 and 6 is accompanied by the liberation to titration of one cationic residue per chain only in the C variant (5). At low temperature, in the pH range 3.7–5.1, the A variant is able to form octamers (7, 8), the B variant undergoes only a weak octamerization reaction and can participate in the formation of hybrid octamers when mixed with the A variant (9). The C variant does not form any octamers detectable by the usual macromolecular techniques (8). These differences have suggested that the Asp ↔ Gly substitution is located in the area of contact between the dimers when they form octamers and that the His ↔ Gln substitution is in the vicinity. Other differences have not yet been explained: The solubility increases from A to C (3, 10); A is more resistant to heat denaturation than B (11) and A is more sensitive to hydrolysis by trypsin than B (12, 13). Such correlations between natural amino acid substitutions and physico-chem-

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ical properties can help to locate residues that play a key role in these properties, as well as to detect some features of the folding of the polypeptide chain.

Recently Grosclaude *et al.* (14) identified another variant of bovine β-lactoglobulin, called D, in two breeds of the Simmenthal type. At pH 8.6 this variant has an electrophoretic mobility lower than the other three variants, whereas the mobilities of the B and D variants are identical at pH 3.1. Chemical and physico-chemical investigations have been undertaken to characterize this variant and are reported in this paper.

MATERIALS AND METHODS

β–Lactoglobulins B and D (β–B, β–D) were prepared from the milk of homozygous cows by the method of Aschaffenburg and Drewry (15) and recrystallized four times. The D variant was prepared from a single cow of the Montbeliard breed.[2] The yield of D variant crystals was considerably lower than that obtained from β–B.

After reduction at room temperature for 1.5 hr in 8 M urea, 0.1 M veronal buffer pH 8.0, and 0.04 M β-mercaptoethanol, the β–lactoglobulin solutions (10 g/liter were alkylated by adding iodoacetic acid to this mixture up to 0.0064 M, the pH being maintained constant at 8.0. After 15 min, the pH was dropped to 5.0 and the solution exhaustively dialyzed against distilled water and freeze dried. The residue was used for chymotryptic digestion. Alkylation by iodoacetamide gave chymotryptic peptides poorly resolved by paper electrophoresis.

Worthington 3× crystallized chymotrypsin, Worthington D.F.P.-treated carboxypeptidase A and leucine aminopeptidase were used.

The chymotryptic digestions were carried out under nitrogen at 38° and pH 8.0 in a pH-stat on 10 ml of a 20 g/liter solution of protein in distilled water with an enzyme-substrate ratio of 1/76 (w/w). When the digestion was essentially complete (after 3 hr), as judged by the uptake of 0.5 N NaOH (ca. 12 equivalents per polypeptide chain), the pH was dropped to 6.5 by adding 0.1 N HCl and the digest was evaporated to dryness under vacuum. The chymotryptic peptides were dissolved in a pH 6.5 acetic acid–pyridine buffer (4/100, v/v made up to 1 liter with water). The insoluble material was centrifuged for 10 min at 1,100*g* and washed four times with the same buffer. The supernates were pooled and dried under vacuum.

The chymotryptic peptides were separated by preparative high-voltage electrophoresis (55 V/cm, 1.5 hr) on Whatman 3 MM paper in the pH 6.5 buffer. Peptides were eluted with a 5% acetic acid solution and purified by preparative chromotography in *n*-butanol/acetic acid/water (200-30-75, v/v), followed, after elution, by electrophoresis under the same conditions as above. The peptides were then subjected to amino acid analysis.

Both difference peptides were treated at 40° with leucine amino-peptidase (enzyme-substrate ratio 1/2 to 1/20) at pH 8.5 in 0.1 M Tris-HCl, 2.5×10^{-2} M $MgCl_2$ buffer. After 19–40 hr the digest was evaporated to dryness, dissolved in 0.2 N citrate buffer, pH 2.2, and subjected to amino acid analysis. The carboxypeptidase A treatment was identical, except that $MgCl_2$ was omitted from the incubation mixtures and the enzyme-substrate ratio used was 2/1 (w/w); the length of the treatment was 2–24 hr.

The amino acid analyses of the β-lactoglobulins were carried out after hydrolysis of freeze-dried products (dried again under vacuum over P_2O_5 for 24 hr.) over periods of 24, 48, 96, and 120 hr in triplicate (except for 120 hr.) using standard conditions (5.7 N HCl, at 110°, under vacuum). The amino acid analyses were performed with a Beckman Unichrom Amino Acid Analyzer according to Moore *et al.* (16). Tryptophan was determined by the spectrophotometric method of Bencze and Schmid (17).

The amino acid analyses of the peptides were carried out following a 24-hr hydrolysis; the absence of tryptophan in the two different peptides was checked by the Ehrlich reagent on paper electrophoregrams of the chymotryptic peptides.

Optical rotatory dispersion measurements were made on a Cary Model 60 spectropolarimeter and the variation of optical rotation with pH was determined on a Jouan Quick polarimeter. The rotatory dispersion parameters a_0 and b_0 of the Moffitt-Yang equation were calculated using $\lambda_0 = 212$ nm and taking a Lorentz factor of 0.79 and a mean residue weight of 110.3. Circular dichroism experiments were performed on a Durrum-Jasco ORD/UV 5 apparatus.[3]

The ultracentrifugal experiments were carried out with a Spinco Model E analytical ultracentrifuge.

The titration curve of β–D was obtained in the

[2] We thank Dr. F. Grosclaude from the Department of Animal Genetic, I.N.R.A., for making available to us the milk of this cow.

[3] Mention of companies or products is for the convenience of the reader and does not constitute an endorsement by the United States Department of Agriculture.

presence of 0.15 M KCl using a Radiometer Model 4 pH meter. The technique was essentially the same as described previously (18); the only modification was that, in the present study, acid or base was added serially to the protein solution, a pH reading being taken after each addition.

RESULTS AND DISCUSSION

Chemical Composition

The amino acids composition of β-lactoglobulin D is reported in Table I; its similarity, within the limits of experimental errors, with that of β-B, given by Piez *et al.* (2) and Kalan *et al.* (3) suggests a replacement of the type: Dicarboxylic acid ↔ amide to explain the difference of electrophoretic mobilities of the two variants.

Paper electrophoresis of a chymotryptic digest of the two variants, B and D, reveals two peptides, B_1 and D_3, migrating toward the anode (Fig. 1) each of which has no counterpart in the other digest. These two peptides were isolated and analyzed (Table II). They have the same amino acid composition, which confirms the conclusion from the amino acid analyses made on the whole protein. Carboxypeptidase A removes from both peptides LeuOH and Ile and leucine amino-peptidase removes from B_1 HVal, Glu, Leu, Gly and from D_3 HVal, Glu, Gln–(or Asn), Leu and Gly (Table III). The N-terminal end of peptide D_3 seems to have one glutamine or asparagine residue more and probably one glutamic acid residue less than peptide B_1. A finger print on paper of a leucine aminopeptidase digest of peptide D_3 (high voltage electrophoresis at pH 1.9 and chromatography in *n*-butanol/acetic acid/water: 200-30-75 v/v) reveals no asparagine, confirming that the replacement is B (Glu) ↔ D (Gln). This difference explains the electrophoretic mobility of variant D and its titration curve (see below). It corresponds to only one nucleotide base replacement, $GA^{A}_{G} \leftrightarrow CA^{A}_{G}$, in the codon. Other differences between the two variants are also possible, such as sequence interchange, double replacement, provided they do not alter the total amino acid composition and the number of ionizable groups.

TABLE I

AMINO ACID COMPOSITION OF β-LACTOGLOBULIN *D*

Amino acids	No. of residues per mole[a]		Integral no.
Asp	15.21	.20[b]	15
Thr	8.00		8
Ser	7.00		7
Glu	24.90	.40	25
Pro	8.00	.09	8
Gly	4.00	.06	4
Ala	15.10	.07	15
½ *Cys*	4.00		4
Val	9.08		9
Met	4.14	.07	4
Ile	9.98		10
Leu	21.03	.24	21
Tyr	4.05	.10	4
Phe	4.05	.08	4
Trp	2.06		2
Lys	14.60	.25	15
His	2.06	.05	2
Arg	3.00	.06	3
NH_3	(12.4)		(12)
mol wt			18,080

[a]Per polypeptide chain of a mol wt of 18,080. *Thr*, *Ser*, NH_3 were extrapolated to zero time, ½ *Cys* value was taken at 24 *hr* and *Val*, *Ile*, *Glu* at 96 *hr*. Other numbers of residues were average values of the four different times of hydrolysis.

[b]Measure of repeatability at the 95% confidence limit.

According to these results, the partial sequences of the two peptides can tentatively be written as:

Peptide B_1 :

HVal-(Glu, *Glu*, Leu, Gly)
107 110 111

(Thr, Glx, Pro, Leu, Lys, Asx, Glx)
117 118 119

Ile-LeuOH

Peptide D_3 :

HVal (Glu, *Gln*, Leu, Gly)
107 110 111

(Thr, Glx, Pro, Leu, Lys, Asx, Glx)
117 118 119

Ile-LeuOH.

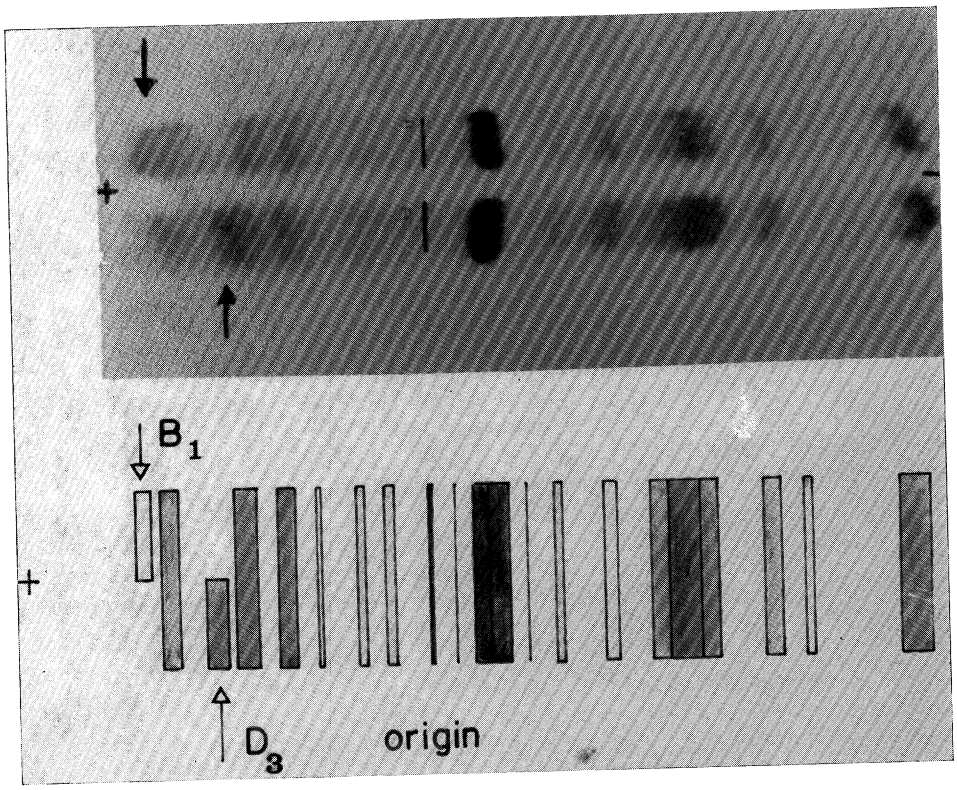

FIG. 1. High-voltage electrophoreses of chymotryptic hydrolyzates of *S*-carboxymethylated β-lactoglobulins B and D at pH 6.5.

Examination of its composition allows the placement of this peptide in position 107–119 in the sequence published by Frank and Braunitzer (19), and, taking their results into account, to propose that the mutation Glu ↔ Gln occurs at position 109. The mobility toward the anode of peptide D_3 at pH 6.5 suggests that at least one of the Glx residues is a glutamic acid residue even if it is assumed that Asx_{118} is an aspartic acid residue in the sequence proposed by Frank and Braunitzer (19). There seems to be a discrepancy in the composition of peptides B_1 and D_3 in Frank and Braunitzer's sequence: The C-terminal Ile-LeuOH does not fit into the proposed sequence. Although these amino acids are obtained in very good yield, it is still possible that a contaminating peptide had not been fully removed by electrophoresis and chromatography on paper. A rather remote possibility would be a sequence interchange common to the two analyzed variants but different than the ones analyzed by Frank and Braunitzer.

The site of the B ↔ D mutation is 12 ± 1 amino acid residues removed from the A (Asp) ↔ B (Gly) mutation, which has been located by Frank and Braunitzer at position 120–122 (19).

Physical Chemical Properties

In order to verify the Glu ↔ Gln amino acid substitution between β-lactoglobulins B and D, an acid-base titration curve of β-D was carried out and compared with that of β-B.[4] The results are shown on Fig. 2. It is evident that, in the acid region, the two titration curves are essentially identical, both tending toward a maximum acid binding capacity of +40. This indicates that the

[4] The titration experiments are taken from the Ph.D. dissertation of J. J. Basch, Temple University, 1968.

TABLE II

AMINO ACID COMPOSITION OF PEPTIDES B_1 AND D_3

Amino acids	Peptide B_1		Peptide D_3	
	μmoles	Average ratios rounded[a]	μmoles	Average ratios rounded
Asp	1.04	1	1.04	1
Thr	0.92	1	0.87	1
Ser	0.01	0	0.009	0
Glu	3.82	4	3.95	4
Pro	1.86	2	1.83	2
Gly	1.10	1	1.18	1
Ala	0.02	0	0.002	0
Cys	0		0	0
Val	1.00	1	0.96	1
Met	0.006	0	*tr.*	0
Ile	0.92	1	0.93	1
Leu	2.85	3	2.90	3
Tyr	0	0	*tr.*	0
Phe	0	0	*tr.*	0
Lys	0.85	1	0.78	1
His	*tr.*	0	*tr.*	0
Arg	*tr.*	0	*tr.*	0

[a]Results are numbers of residues calculated in considering the average value of μmoles obtained for *Asp*, *Gly* and *Lys* as representing one residue.

TABLE III

N- AND *C*-TERMINAL ENDS OF PEPTIDES B_1 AND D_3

LAP	B_1 *Val* (1.00),[a] *Glu* (0.42), *Gln-Asn* (0), *Gly* (0.36), *Leu* (0.28)
	D_3 *Val* (1.00), *Glu* (0.34), *Gln-Asn* (0.23), *Gly* (0.41), *Leu* (0.28)
CPA	B_1 *Leu* (1.00), *Ile* (0.58)
	D_3 *Leu* (1.00), *Ile* (0.77)

[a]Figures in parentheses indicate the number of residues which are liberated by leucine aminopeptidase (*LAP*) and carboxypeptidase *A* (*CAP*) after 24 hr, *Val* and *Leu* respectively being taken equal to 1.

two variants have identical numbers of cationic residues in agreement with the amino composition (see Table I). Above pH 3.5, a divergence becomes evident, with the β-D curve lying above that of β-B. At pH 6.5 the difference becomes two groups per 35,500 daltons molecular weight, β-D binding fewer protons than the B variant. This difference in the titration curves supports the conclusion that β-D has one less anionic group per subunit than β-B. The same conclusion is consistent with the displacement of the β-D isoionic point (in 0.15 M KCl) to pH 5.40 from β-B at pH 5.25. Further examination of the β-D curve reveals the presence of the two abnormal carboxyls which ionize between pH 7 and 8, just as in the other three variants, as well as in the goat protein (4, 18, 20–26). Thus, the titration curve fully supports the results of the amino acid analysis and difference peptide studies.

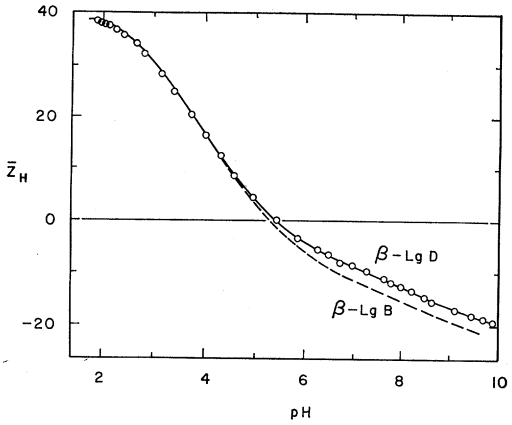

FIG. 2. Titration curve of β-lactoglobulin D in 0.15 M KCl. The dashed line represents the titration curve of β-B.

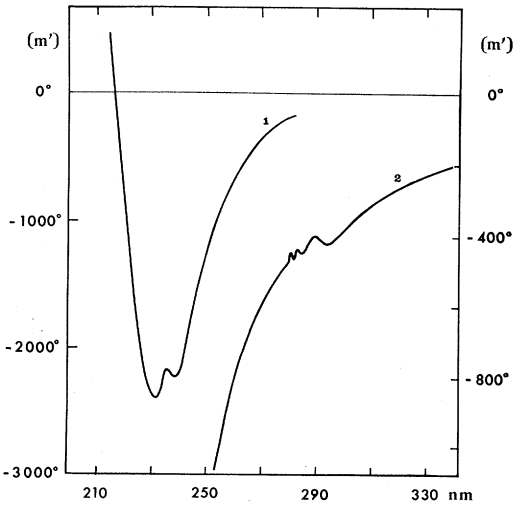

FIG. 3. Optical rotatory dispersion of β-lactoglobulin D at pH 7.5 (1-cm cell). Curve 1: 0.13 mg/ml; scale of [m'] at the left. Curve 2: 1.4 mg/ml; scale of [m'] at the right.

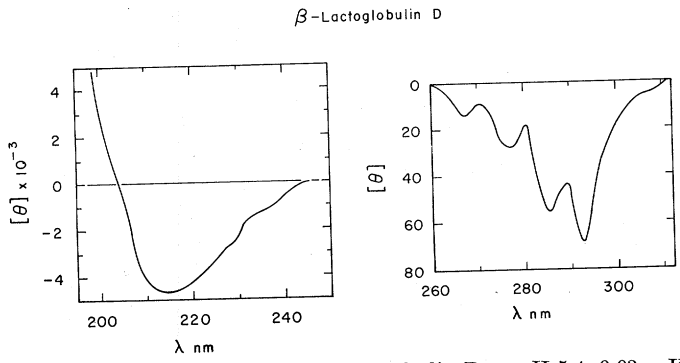

FIG. 4. Circular dichroism spectrum of β-lactoglobulin D at pH 5.4, 0.03 M KCl, 25°.

The optical rotatory dispersion curve of β-D was obtained at room temperature in phosphate buffer, pH 7.5, 0.1 ionic strength. The results are shown on Fig. 3. The D variant shows two troughs at 231 ± 1 nm and 237 ± 1 nm and a small maximum around 234 ± 1 nm, as had been found with the other three variants (4); the values of the residue rotation [m′] in the troughs, −2,200−−2,400°, are in good agreement with those reported for variants A, B, and C. The crossover point is in the vicinity of 220 nm. Just as the other three variants, β-D also displays weak, but significant aromatic Cotton effects between 280 and 300 nm. A Moffitt-Yang plot uncorrected for side effects leads to a value at pH 7.5 of $a_0 = -234°$ and $b_0 = -95°$.

The infrared spectrum in the amide I region showed that β-D does not differ essentially from the other variants in this respect. The peak maximum is located at 1,635 cm^{-1}, suggesting the presence of β-structure in this variant as well (27).

The circular dichroism spectrum of β-D, obtained at pH 5.4 in 0.03 M NaCl is shown in Fig. 4. Just as with variants A, B, and C (28, 29), and the goat protein (25), this spectrum is characterized by negative absorption between 208 and 245 nm, with a maximum at 215–216 nm and a weak shoulder at 236 nm. Between 260 and 310 nm, the CD spectrum is characterized by at least five bands, with maxima around 267, 278, 285, 293, and 307 nm. Just as in the other variants (28), the 293 and 285 maxima may be attributed to tryptophan transitions (30), as may be the band at 267 nm (30), although contributions from tyrosines and disulfides are not excluded. The 278-nm band probably corresponds to tyrosine transitions (31). The band at 307 nm may reflect either the exciton splitting of a tryptophan transition (32), or possibly a disulfide transition (33). In general, the positions and intensities of the bands in both spectral regions are similar to those of β-B (28), indicating a close similarity in the conformations of the two proteins.

The conformational transition at alkaline pH known to exist in β-A, B, and C (20, 4), is also undergone by the D variant. This is shown in Fig. 5 where the optical rotations of the B and D variants are presented as a function of pH between pH 5 and 10. It is

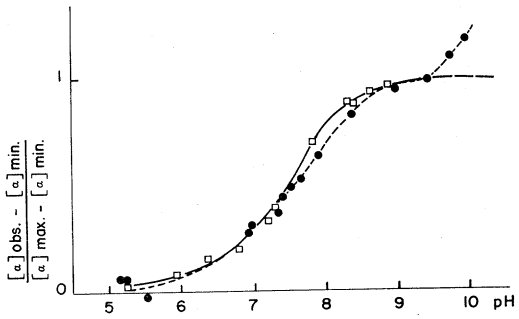

FIG. 5. Conformational transitions of β-lactoglobulins D and B at alkaline pH. Protein concentration: 4.2 mg/ml; 20°; 0.1 M NaCl. The pH was adjusted by addition of small aliquots of concentrated NaOH. The optical rotatory power was followed at 395 nm for variant D (●) where $[\alpha]_{395}$ varies from −72 to −120°, and at 436 nm for variant B (□) where $[\alpha]_{436}$ varies from −65 to −106°. The ordinates are expressed in relative value.

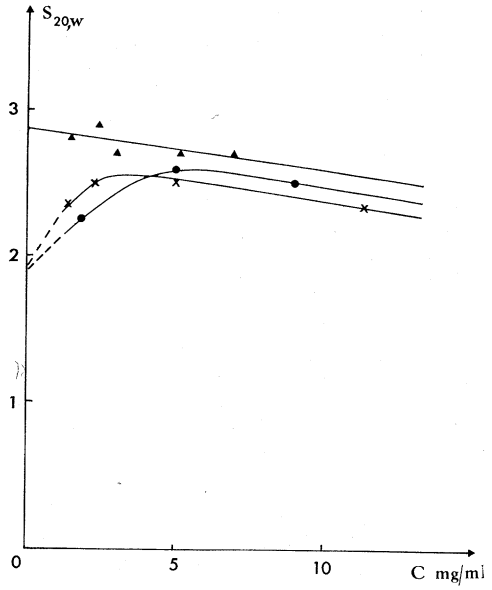

FIG. 6. Concentration dependence of the sedimentation coefficients of β-lactoglobulin D at various pH's. Experiments at pH 5.2 (▲) and 2.4 (×) were in HCl, 0.1 M NaCl and at pH 8.6 in veronal buffer, Γ/2 − 0.1 (●).

evident that, just as in the titration, β-D undergoes this transition with a pH dependence that indicates the ionization of one group per subunit. In both proteins, the midpoint occurs at pH 7.5–7.6.

Ultracentrifugal studies carried out with the D variant show that at low temperature (4°), at pH 4.5 (0.1 M acetate buffer) and a protein concentration of 17 mg/ml, β-lactoglobulin D shows one symmetrical peak ($s_{20,w}$ = 2.6S). Thus, just as the B and C variants which also lack the extra aspartic acid residue present in A, β-D does not form octamers in any significant amount.

On both sides of the isoelectric point, a single and slightly asymmetric peak is found. The values of the sedimentation coefficients are plotted as a function of protein concentration in Fig. 6. These results clearly indicate that a dimer ⇌ monomer equilibrium exists in this variant as well at both acid and alkaline pH's (7, 34).

At pH 5.1 and at 20°, the plot of the sedimentation coefficient extrapolates to a value of $s_{20,w}$ = 2.9S, very similar to the one found for the other three variants.

Hybridization experiments between β-B and β-D were carried out as described before for the other variants (8, 35).[5] The electrophoretic patterns obtained were identical whether the proteins were adjusted to low pH individually or in a mixture. While this does not establish the formation of β-B-D hybrids, neither does it disprove such formation. The earlier interpretation that lack of formation of a new moving boundary, intermediate between the two variants, demonstrates lack of hybrid formation is now open to question in view of Hill's analysis of such systems (36). Hill has concluded that, if any dissociation into subunits is possible at the conditions of the electrophoretic experiments, then a hybridizing system may display a pattern very similar to that formed by a mixture of two noninteracting variants. The free energy of association between the β-lactoglobulin subunits at the pH of the electrophoretic experiments (pH 5.3–5.6) is in the vicinity of −10 kcal/mole (7). Thus, very weak, but finite dissociation of the subunits is possible even in the isoelectric region, making the interpretation of hybridization experiments uncertain.

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REFERENCES

1. GORDON, W. G., BASCH, J. J., AND KALAN, E. B., *J. Biol. Chem.* **236**, 2908 (1961).
2. PIEZ, K. A., DAVIE, E. W., FOLK, J. E., AND GLADNER, J. A., *J. Biol. Chem.* **236**, 2912 (1961).
3. KALAN, E. B., GREENBERG, R., AND WALTER, M., *Biochemistry* **4**, 991 (1965).
4. TIMASHEFF, S. N., TOWNEND, R., AND MESCANTI, L., *J. Biol. Chem.* **241**, 1863 (1966).
5. TIMASHEFF, S. N., MESCANTI, L., BASCH, J. J., AND TOWNEND, R., *J. Biol. Chem.* **241**, 2496 (1966).
6. JENNESS, R., PHILLIPS, N. I., AND KALAN, E. B., *Federation Proc.* **26**, 476 (1967).
7. TIMASHEFF, S. N. AND TOWNEND, R., *J. Am. Chem. Soc.* **83**, 464 (1961).

[5] We thank Dr. Robert Townend for carrying out the hybridization experiments.

8. Townend, R., Herskovits, T. T., Swaisgood, H. E., and Timasheff, S. N., *J. Biol. Chem.* **239,** 4196 (1964).
9. Kumosinski, T. F. and Timasheff, S. N., *J. Am. Chem. Soc.* **88,** 5635 (1966).
10. McMeekin, T. L. and Treece, J. M., *Federation Proc.* **23,** 474 (1964).
11. Gough, P. and Jenness, R., *J. Dairy Sci.* **45,** 1033 (1962).
12. Preaux, G., Archambeau, J., Delanghe, A., and Lontie, R., *Arch. Intern. Physiol. Biochim.* **70,** 752 (1962).
13. Monnot, M., *Biochim. Biophys. Acta* **93,** 31 (1964).
14. Grosclaude, F., Pujolle, J., Garnier, J., and Ribadeau-Dumas, B., *Ann. Biol. Animale, Biochim. Biophys.* **6,** 215 (1966).
15. Aschaffenburg, R. and Drewry, J., *Biochem. J.* **65,** 273 (1957).
16. Moore, S., Spackman, D. N., and Stein, W. H., *Ann. Chem.* **30,** 1185 (1958).
17. Bencze, W. L. and Schmid, K., *Ann. Chem.* **29,** 1193 (1957).
18. Basch, J. J. and Timasheff, S. N., *Arch. Biochem. Biophys.* **118,** 37 (1967).
19. Frank, G. and Braunitzer, G., *Hoppe-Seylers Z. Physiol. Chem.* **348,** 1691 (1967).
20. Tanford, C. and Taggart, V. G., *J. Am. Chem. Soc.* **83,** 1634 (1961).
21. Pantaloni, D., *Compt. Rend. Acad. Sci.* **252,** 2459 (1961).
22. Pantaloni, D., *Compt. Rend. Acad. Sci.* **256,** 4991 (1963).
23. Pantaloni, D., *Ph.D. Dissertation,* Paris, 1965.
24. Nozaki, Y., Bunville, L. G., and Tanford, C., *J. Am. Chem. Soc.* **81,** 5523 (1959).
25. Townend, R. and Basch, J. J., *Arch. Biochem. Biophys.* (**126,** 59 (1968).
26. Ghose, A. C., Chaudhuri, S., and Sen, A., *Arch. Biochem. Biophys.* **126,** 232 (1968).
27. Susi, H., Timasheff, S. N., and Stevens, L., *J. Biol. Chem.* **242,** 5460 (1967).
28. Townend, R., Kumosinski, T. F., and Timasheff, S. N., *J. Biol. Chem.* **242,** 4538 (1967).
29. Timasheff, S. N., Susi, H., Townend, R., Stevens, L., Gorbunoff, M. J., and Kumosinski, T. F., *in* "Conformations of Biopolymers" (G. N. Ramachandran, ed.), p. 173, Academic Press, New York (1967).
30. Stevens, L., Townend, R., Timasheff, S. N., Fasman, G. D., and Potter., J, *Biochemistry,* **126,** 3717 1968).
31. Beychok, S. and Fasman, G. D., *Biochemistry* **3,** 1519 (1964).
32. Gratzer, W. B., *in* "Poly-α-Amino Acids" (G. D. Fasman, ed.), p. 177, Dekker, New York (1967).
33. Beychok, S., *in* "Poly-α-Amino Acids" (G. D. Fasman, ed.), p. 293, Dekker, New York (1967).
34. Georges, C. and Guinand, S., *Biochim. Biophys. Acta* **59,** 737 (1962).
35. Townend, R., Kiddy, C. A., and Timasheff, S. N., *J. Am. Chem. Soc.* **83,** 1419 (1961).
36. Hill, R. J., *Brookhaven Symp. Biol.* No. 17, p. 201, 1964.